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Award Number: W81XWH-09-1-0399

TITLE: Mechanism of Prostate Cancer Prevention by Down-Regulation of the GH/IGF Axis

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REPORT DATE: July 2012

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
July 2012	Annual	1 July 2011 – 30 June 2012

4. TITLE AND SUBTITLE

Mechanism of Prostate Cancer Prevention by Down-Regulation of the GH/IGF Axis

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-09-1-0399

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

University of Illinois
Chicago, IL 60612-4305

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The purpose of this project was to test the hypothesis that growth hormone (GH) stimulates specific pathways, some of which are independent of IGF-I, for promoting proliferation and inhibiting death in prostate cancer cells. Our first aim is to determine which of the multiple signaling pathways stimulated by GH receptor are required to promote prostate cancer. Our strategy was to cross mice that develop prostate cancers due to a large T antigen (TAg) transgene with mice that lack discrete segments of the intracellular portion of the GH receptor. We have not yet completed this experiment due to insufficient breeder fecundity. To assess the relative contribution of IGF-I and GH to prostate carcinogenesis, we grafted prostate tissue harboring the TAg transgene. The grafts were either Ghr+/+ or Ghr-/- and therefore were able to respond to IGF-I but not detect GH. Our results suggest that IGF-I is the major driver of carcinogenesis. We also planned to propagate human prostate cancer cells in vitro and expose them to a human growth hormone antagonist. In vitro, however, the cells were neither stimulated by recombinant human GH nor inhibited by GH antagonist.

15. SUBJECT TERMS
Growth hormone, growth hormone antagonist, prostate cancer, cancer prevention.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	13	19b. TELEPHONE NUMBER *(include area code)*

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Introduction

It has been known for many years that androgen ablation can reverse the course of the prostate cancer and this has formed the foundation of therapy for decades. Invariably, however, androgen resistant forms emerge that resume disease progression. In recent years, additional pathways have been identified that may provide alternative targets to androgen signaling. One such pathway involves the growth hormone/insulin-like growth factor-I (GH/IGF-I) axis. In vitro and in vivo studies of rodent and primate model systems illustrate that GH and IGF-I can induce prostate epithelial cell proliferation and differentiation while blocking apoptosis. Recent clinical trials indicate that elevated circulating IGF-I confers an increased risk for the development of prostate cancer. Our hypothesis was that GH stimulates specific pathways, some of which are independent of IGF-I, for promoting proliferation and inhibiting apoptosis in prostate cancer cells. Our first aim was to determine what signaling pathways stimulated by GH and its receptor are required to promote prostate cancer. We crossed the C3(1)/TAg mouse, which develops prostate cancers, with mice that have defined deletions in their GH receptor that stimulate specific signaling pathways. We had hoped that these experiments would tell us which GH-stimulated pathways are most important in supporting carcinogenesis. Due to the lack of fecundity of the breeder mice, however, we have not yet generated a sufficient quantity of bigenic mice to complete the experiment. Another experiment in Aim 1 was to compare the relative contributions of GH and IGF-I to tumor growth. We generated mice that harbored one copy of the TAg transgene and were homozygous for either the wild-type ($Ghr^{+/+}$) or knockout ($Ghr^{-/-}$) of Ghr. Prostates of these mice were then transplanted into immunodeficient mice where they could grow in the presence of normal GH and IGF-I serum titers. We observed that prostate carcinogenesis proceeded similarly in the $Ghr^{-/-}$ prostates as in the $Ghr^{+/+}$ prostates indicating that IGF-I signaling is the dominant pathway driving carcinogenesis in this system. Our second aim was to determine which pathways are involved in cancer regression caused by GH removal or antagonism. We used the GH antagonist developed by our collaborator, Dr. John Kopchick, to study the mechanisms that GH antagonists can kill prostate cancer cells resulting in tumor regression.

Body

Below is a description of the research accomplishments associated with each task outlined in the approved Statement of Work.

Task 1. To determine what signaling pathways stimulated by GH and its receptor are required to promote prostate cancer.

a. Animal protocol reviewed (ACURO; months 1 - 2)
b. Cross C3(1)/TAg mice with GHR mutant mice to generate 5 groups of 30 mice each (months 3 - 18).
c. Conduct PCR analysis of mouse tail snips for genotyping (months 3 - 18)
d. Sacrifice mice for necropsy and histology of prostate glands (months 18 - 26).
e. Histologic analysis of slides, measurement of prostate lesions (months 26 - 29
f. Data analysis and report writing (months 29 - 36)

The rationale for Task 1 is based on our previous studies demonstrating that mice null for the GH receptor ($Ghr^{-/-}$) were nearly refractory to TAg induced prostate carcinogenesis [1]. The GHR gene-disrupted mouse ($Ghr^{-/-}$), which has less than 10% of the plasma IGF-I found in GHR wild-type mice [2], was crossed with the C3(1)/Tag mouse, which develops PIN driven by TAg that progress to invasive prostate carcinoma in a manner similar to the process observed in humans [3]. Progeny of this cross were genotyped and TAg/ $Ghr^{+/+}$ and TAg/$Ghr^{-/-}$ mice were sacrificed at 9 months of age. Seven of 8 TAg/$Ghr^{+/+}$ mice harbored PIN lesions of various grades. In contrast, only one of the 8 TAg/$Ghr^{-/-}$ mice exhibited atypia [1]. Disruption of the *Ghr* gene altered neither

prostate androgen receptor expression nor serum testosterone titers. Expression of the Tag oncogene was similar in the prostates of the two mouse strains. Immunohistochemistry revealed a significant decrease in prostate epithelial cell proliferation and an increase in basal apoptotic indices [1]. These results indicate that disruption of GH signaling significantly inhibits prostate carcinogenesis.

Activation of the GH receptor by binding GH results in the initiation of several distinct downstream signals. The purpose of the experiments in Task 1 is to study the role of each pathway in prostate carcinogenesis. The pathways activated by GH receptor including STATs 1, 3 and 5a/b, phosphoinositide Akt, Src, extracellular-signal regulated kinase (ERK), c-Jun N-terminal kinase and stress activated protein kinase pathways, and increased cell calcium, demonstrable *in vitro*. Our collaborator, Dr. Michael Waters, has engineered mice with targeted mutations in their cytoplasmic domains to eliminate key signaling pathways [4]. Thus far, three such mutant mice have been engineered (Figure 1). One mouse has its GHR truncated at residue 391, removing all STAT5 generation and presumably other signals in the central segment of the cytoplasmic domain. A second mouse has its GHR truncated at 569 with the two distal tyrosines converted to phenylalanine to remove the majority of STAT5 signaling and the SHP2-binding site, originating from the distal 80 residues of the intact receptor. Finally, a third mouse harbors a mutation of the box 1 sequence to abrogate JAK2 activation, which should leave the Src family kinase intact, and indicate the extent of GH action through JAK2.

We have obtained these mice from our collaborator, Dr. Waters, and completed the initial crossed between the C3(1)TAg mice, which contribute the prostate cancer inducing oncogene, with the GH receptor truncation mice (GHR; Table 1). We are now conducting the hybrid production crosses outlined in Table 2. Our current progress in producing experimental animals for Task 1 is summarized in Table 3. Our target group size was 30 mice for each group, but we have thus far generated a much lower number due to unexpectedly poor fecundity in our breeders. The productivity of the breeders reflects the degree of GH receptor truncation: we have the most pups in the M569 group, which has the smallest truncation, and the fewest pups in the Box1 group, which has the greatest amount of the receptor missing. Two other features of this cross are curious (Figure 2 and Table 4). We observed in this breeding plan that in all groups (m569, m391 and Box1) mice harboring the SV40 oncogene weigh substantially less than mice lacking this transgene. We did not observe this phenomenon in our previously published work in which we crossed the C3(1)/TAg mice with mice lacking any GH receptor at all [1]. Our only explanation for this discrepancy is that in the published work, the C3(1)/TAg mice (FVB background) were crossed with $Ghr^{-/-}$ mice with a background of Ole/Balb/c. In the current studies, the Ghr mutant mice are of the C57/BL background. This decrease in body weight may contribute to the low number of mice we observe in the TAg positive mice that are also homozygous for each of the mutations (m569, m391 and Box1). As clearly shown in Table 3, we are not observing the expected Mendelian distribution of mice. Dispite our breeding issues, we are generating mice in each group and given the potential knowledge to be gained by this experiment, we hope that we can, within the next reporting period, generate enough mice to address the questions posed in Task 1.

Task 2. The purpose of this task is to compare the relative contributions of GH and IGF-I to tumor growth.

a. Breed mice bearing TAg and either $Ghr^{+/+}$ (N=15) or $Ghr^{-/-}$ (N=15) (months 3 - 6)
b. Genotype pups by PCR (months 3 - 8)
c. Transplant 3-day-old prostates under kidney capsule of immunodeficient recipients (months 3 - 8)
d. Sacrifice hosts (months 11 - 17)
e. Process tissues and analyze lesions using image analysis software (months 18-24)

We have completed Task 2. Our published [1] and preliminary data show that when GH signaling is disrupted, prostate carcinogenesis is inhibited. However, whenever GH is inhibited, IGF-I, which is under the control of GH, also is down-regulated. Therefore, it was not clear if carcinogenesis was blocked due to the lack of GH signaling or IGF-I signaling. To address this question, we transplanted prostates from $Ghr^{-/-}$ mice, which

develop few prostate cancers but have low IGF-I, to immunodeficient mice with normal GH and IGF-I levels. Our control experiment had prostates from *Ghr*$^{+/+}$ mice transplanted under the capsules of the contralateral kidneys of the same mice as the *Ghr*$^{-/-}$ transplants. If GH was the critical driver of prostate carcinogenesis in this system, we would expect to see few preneoplastic lesions in the transplants that lacked GH receptor. However, we observed a similar degree of neoplasia develop in both sets of transplants indicating that IGF-I, not GH, is critical for cancer development (Figure 5).

Task 3. To determine what pathways are involved in cancer regression caused by GH removal or antagonism.

a. Conduct site-directed mutagenesis of the human GH cDNA that changes the glycine codon at position 120 to one encoding lysine (months 3 - 6).
b. Purify human and mouse GH from inclusion bodies (months 6 - 36).
c. Scale up production of the E. coli cultures producing mouse and human GH antagonists (months 6 - 36).
d. Scale up purification of human and mouse GH antagonists from E. coli cultures (months 6 – 36). Deliverables are purified human and mouse GH antagonists for use in the proposed studies.
e. Treat human (LNCaP & PC-3) and mouse (Pr-117) prostate cancer cells with GHA in vitro (months 9 - 30).
f. Cross C3(1)/TAg mice with GHA mice heterozygous for the C3(1)/TAg oncogene and either heterozygous for the GHA transgene or null for this transgene (months 37 – 40).
g. Sacrifice mice (months 46 - 48).
h. Analyze prostate tissues for activity of GH related pathways (months 46 - 48)
i. Prepare report of results.

In last report, we detailed the successful experiments by our collaborator, Dr. Kopchick of Ohio University, who has provided us with recombinant human GH and recombinant human GH antagonist (GHA; Task 3a through 3d). Dr. Kopchick's laboratory discovered the first GHA [5], which was eventually developed into the FDA-approved drug pegvisomant [6]. We used these materials to evaluate the sensitivity of LNCaP and PC-3 cells to GH or GHA (Task 3e). However, neither GH nor the GHA were respectively able to stimulate or inhibit proliferation of LNCaP or PC-3 cells. We interpreted these results as follows. Human LNCaP or PC-3 cells propagated in culture have been selected to be independent of GH. The serum used in laboratories around the world is non-primate serum (*e.g.,* bovine, equine or porcine). The GHs derived from these species are well known <u>not</u> to stimulate the human GH receptor. Thus, human cancer cells propagated in the most common forms of media proliferate in the absence of GH signaling and do not respond to recombinant human GH or GH antagonist.

To address this problem, we have been granted approval for the following experiments. The Kopchick laboratory has made available to us mice that harbor bovine GH antagonist transgene. This antagonist can inhibit GH signaling in murine cells. To test the hypothesis posed in this Task, we are crossing C3(1)/TAg mice with mice harboring a transgene for the bovine GHA. These experiments will allow us to circumvent the issue of GH non-responsiveness in cultured cells while addressing the central question posed in Task 3. By comparing the prostates of SV40 heterozygous mice that express the antagonist with SV40 heterozygous mice lacking the antagonist (*i.e.,* normal GH signaling) we can asses which signal transduction pathways are key for GH action in cancer.

Key Research Accomplishments

- We have established that proliferation of the human prostate epithelial cell lines designated LNCaP and PC-3 is not affected by either recombinant human growth hormone or the human GH antagonist G120R.
- We have established that the progression of prostate carcinogenesis in our model can be sustained by IGF-I signaling in the absence of GH signaling.

Reportable Outcomes

There were no reportable outcomes during the last reporting period.

Conclusion

The major purpose of this project is to better understand how the GH/IGF-I axis can regulate prostate carcinogenesis. The results of our experiments outlined in Task 2 above suggest that IGF-I, rather than GH, is the major driver of carcinogenesis in this model system. These results are consistent with the findings of many other labs that have proposed that IGF-I is key to cancer development [7]. This finding also corroborates the data of Efstratiadis and colleagues [8] who, studying the growth of mice with GH and/or IGF-I receptors knocked out, concluded that IGF-I is the major factor governing postnatal growth in the mouse. Nevertheless, since GH is the major regulator of the expression of IGF-I and its binding proteins, GH remains an important target to control serum and local IGF-I concentrations. This relationship is even more significant with the disappointing results of recent clinical trials of small molecules and antibodies designed to inhibit IGF-I or its receptor [7]. Thus, even though our results indicate that IGF-I has a greater effect on tumor growth than GH alone, GH is still a key anticancer drug target.

References

[1] Wang Z, Prins GS, Coschigano KT, Kopchick JJ, Green JE, Ray VH, et al. Disruption of growth hormone signaling retards early stages of prostate carcinogenesis in the C3(1)/T antigen mouse. Endocrinology 2005;146:5188-96.

[2] Zhou Y, Xu BC, Maheshwari HG, He L, Reed M, Lozykowski M, et al. A mammalian model for Laron syndrome produced by targeted disruption of the mouse growth hormone receptor/binding protein gene (the Laron mouse). Proc Natl Acad Sci U S A 1997;94:13215-20.

[3] Shibata MA, Ward JM, Devor DE, Liu ML, Green JE. Progression of prostatic intraepithelial neoplasia to invasive carcinoma in C3(1)/SV40 large T antigen transgenic mice: histopathological and molecular biological alterations. Cancer Res 1996;56:4894-903.

[4] Rowland JE, Lichanska AM, Kerr LM, White M, d'Aniello EM, Maher SL, et al. In vivo analysis of growth hormone receptor signaling domains and their associated transcripts. Mol Cell Biol 2005;25:66-77.

[5] Chen WY, Wight DC, Wagner TE, Kopchick JJ. Expression of a mutated bovine growth hormone gene suppresses growth of transgenic mice. Proc Natl Acad Sci U S A 1990;87:5061-5.

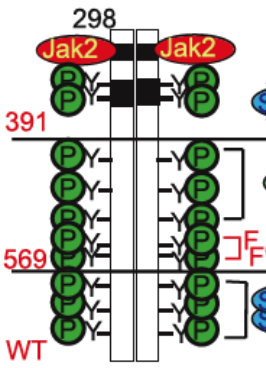
[6] Kopchick JJ, Parkinson C, Stevens EC, Trainer PJ. Growth hormone receptor antagonists: discovery, development, and use in patients with acromegaly. Endocr Rev 2002;23:623-46.

[7] Pollak M. The insulin and insulin-like growth factor receptor family in neoplasia: an update. Nat Rev Cancer 2012;12:159-69.

[8] Lupu F, Terwilliger JD, Lee K, Segre GV, Efstratiadis A. Roles of growth hormone and insulin-like growth factor 1 in mouse postnatal growth. Dev Biol 2001;229:141-62.

Table 1. Initial C3(1)/TAg x GHR Cross

Female (T/t + G/G) x Male (T/T + g/g)



	Signalling			GH-dependent growth	Regulation of metabolic pathways	Examples of genes
	STAT5	JAK2	ERK1/2			
391	0%	100%	100%	11%	Transcription factors regulating metabolic pathways Protein turnover	*PPARγ* *Psmb5,6* *Psmc1,3*
569	30%	100%	100%	44%	Steroid metabolism Proteases Glutathione metabolism	*Hsd3b3* *Serpina6* *Gstm6*
WT	70%			100%	Sulfate metabolism Catecholamine metabolism Amino acid metabolism Lipid metabolism	*Sultn* *Comt* *Csad* *Fabp5*

Figure 1. Summary of the changes in signaling and GH-dependent growth in GHR mutant mice. WT mice enjoy 100% signaling through all major pathways, which contributes to 100% of GH-dependent growth. In mutant 569 mice, however, the loss of 70% of STAT5 signaling results in the loss of 66% of GH-dependent growth. The absence of an active STAT5 pathway in mutant 391 is not adequate to reduce GH-dependent growth to zero; these mice retain 11% of this growth. (From Rowland et al, Mol Cell Biol 25: 66, 2005)

Table 2. C3(1)/TAg /GHR Mutant Hybrid Production Cross
t/T + G/g x T/T + G/g

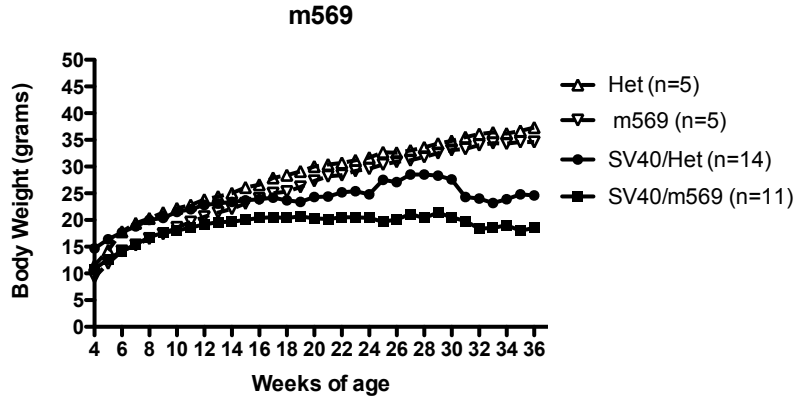
		Genotype			
Class	Proportion	TAg		GHR	Animal Usage
A	1/8	T/T	+	G/G	
B	2/8	T/T	+	G/g	Breeding
C	1/8	T/T	+	g/g	Breeding
D	1/8	t/T	+	G/G	Males: + controls; females: breeders
E	2/8	t/T	+	G/g	Breeding
F	1/8	t/T	+	g/g	Experimental group

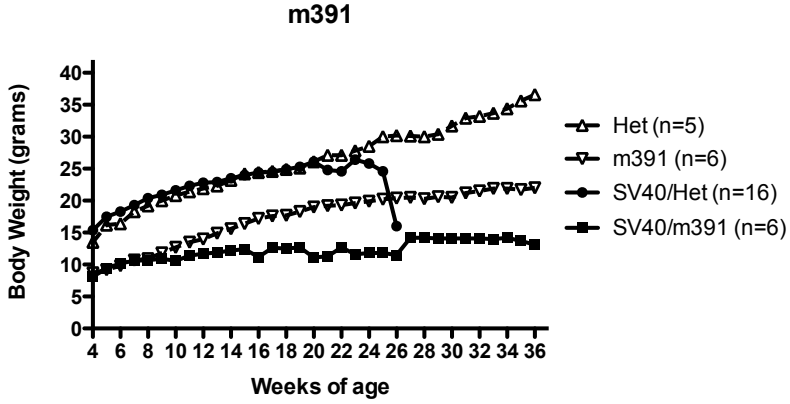
Pups that will be used for the carcinogenesis studies are of the 'D' and 'F' class highlighted above. T: lacking C3(1)/TAg; t carrying C3(1)/TAg; G: GHR wild type; g: GHR mutant.

Table 3. Number of Test Mice Produced for Task 1.

M569		M391		Box1	
Homozygous	Heterozygous	Homozygous	Heterozygous	Homozygous	Heterozygous
11	14	6	16	2	14

Figure 2. Graphical representation of the updated body weights of each of the sets of knockin mice for Task 1 and their respective heterozygous control groups. The total number of mice in each group is noted in parentheses. Note that for each group, the mice harboring SV40 weigh less than their littermates lacking this oncogene.





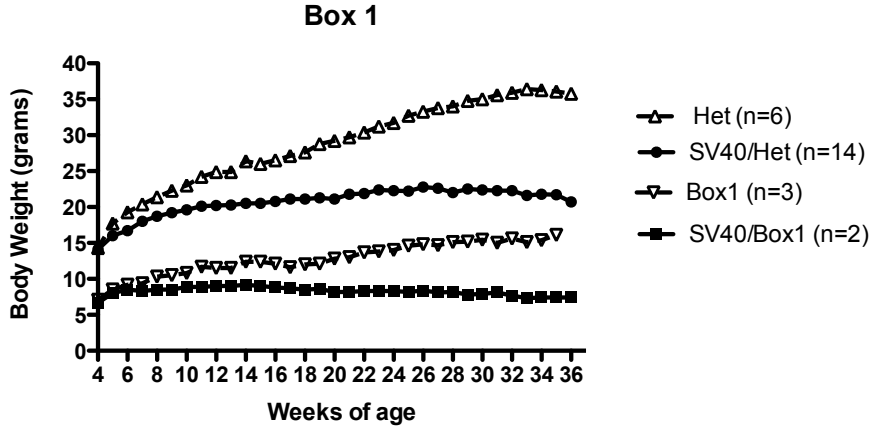


Table 4. Summary of birth dates and sacrifice dates for mice generated as part of Task 1.

m569 heterozygous		**m569 homozygous**	
Birth	*Sacrifice*	*Birth*	*Sacrifice*
12/30/10	09/08/11	03/01/11	11/08/11
02/21/11	10/31/11	03/01/11	11/08/11
02/21/11	10/31/11	03/20/11	11/27/11
02/21/11	10/31/11	09/12/11	05/21/12
03/01/11	11/08/11	09/12/11	05/21/12
03/01/11	11/08/11	09/12/11	05/21/12
09/12/11	05/21/12	10/05/11	06/13/12
09/12/11	05/21/12	10/05/11	06/13/12
10/06/11	06/14/12	10/06/11	06/14/12
10/06/11	06/14/12	11/22/11	07/31/12
11/22/11	07/31/12	11/22/11	07/31/12
12/12/11	08/20/12		
12/12/11	08/20/12		
12/12/11	08/20/12		

m391 heterozygous		**m391 homozygous**	
Birth	*Sacrifice*	*Birth*	*Sacrifice*
03/27/11	12/04/11	02/07/11	10/17/11
12/31/11	09/09/12	03/27/11	12/04/11
01/12/12	09/21/12	02/07/12	10/17/12
01/23/12	10/02/12	02/07/12	10/17/12
01/23/12	10/02/12	03/01/12	11/08/12
02/07/12	10/17/12	04/22/12	12/30/12
02/15/12	10/25/12		
02/19/12	10/29/12		
02/19/12	10/29/12		
02/19/12	10/29/12		
03/01/12	11/08/12		
03/01/12	11/08/12		
03/03/12	11/10/12		
03/29/12	12/06/12		
03/29/12	12/06/12		
04/20/12	12/28/12		

Box 1 heterozygous		**Box1 homozygous**	
Birth	*Sacrifice*	*Birth*	*Sacrifice*
02/12/11	10/22/11	09/10/11	05/19/12
02/12/11	10/22/11	10/15/11	06/23/12
02/12/11	10/22/11		
02/12/11	10/22/11		
02/15/11	10/25/11		
02/15/11	10/25/11		
02/15/11	10/25/11		
04/17/11	12/25/11		
04/17/11	12/25/11		
04/29/11	01/06/12		
04/29/11	01/06/12		
09/10/11	05/19/12		
09/17/11	05/26/12		
10/04/11	06/12/12		

Figure 3. A mouse prostate ready for transplantation.

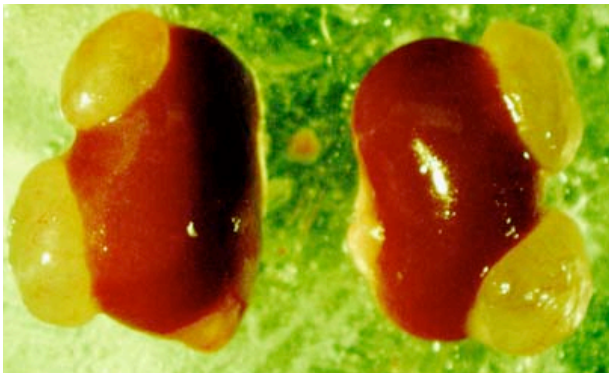


Figure 4. Mouse prostates transplanted under the kidney capsules (2 per capsule) of each nude recipient.

Figure 5. Examples of histology found in *Ghr*$^{+/+}$ and *Ghr*$^{-/-}$ prostates propagated under the kidney capsules of immunodeficient mice with normal IGF-I serum levels. Note that prostate carcinogenesis has progressed to a similar degree in the two groups of prostate tissue. These results suggest that prostate carcinogenesis is not dependent on GH signaling in the presence of normal IGF-I levels.